Molecular Characterization and Evaluation of Probiotic Properties of *Bifidobacteria*

SYNOPSIS

For the award of degree of

Doctor of Philosophy

ZOOLOGY

SUBMITTED BY

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INTRODUCTION

General Classification:

Phylum: Actinobacteria

Class: Actinobacteria

Subclass: Actinobacteridae

Order: *Bifidobacteria*les

Family: *Bifidobacteria*ceae

Genus: *Bifidobacterium*

Bifidobacteria, were initially isolated from the faeces of breast-fed infants (Tisser, 1899). They are gram-positive, non-motile, non-spore forming, strictly anaerobic intestinal rods of variable morphology. According to 9th Edition of Bergey's Manual Systematic Bacteriology, *Bifidobacterium* earlier known as *Bacillus bifidus*, were classified into 29 variant species (Scardovi, 1986). Now more than 48 species have been identified (Ventura *et al.,* 2006; Killer *et al.,* 2011; Endo *et al.,* 2012; Tsuchida *et al.,* 2014).

These microbes can be isolated from various ecological niches, such as sewage, fermented milk and anaerobic environment especially gastrointestinal tracts of humans and animals.

The bacterium shows its optimum growth at temperatures between 36°C and 38°C and a pH range of 6.5 to 7. However, temperature below 25°C and above 45°C inhibits their growth (Shah, 2000). Fructose-6-phosphoketolase, a key enzyme possessed by the microbe produces lactic acids and acetic acids from carbohydrates without the production of CO_2 (Scardovi, 1986). The presence of this enzyme in the organism is an important test to confirm its identification (Scardovi, 1986).

Complex pathogenic micro-organisms also inhabit the gastrointestinal tract of various animal species (Walter, 2008). *Bifidobacterium* are the most common genera present in the GIT of animals and have been linked with good health of the host, reducing the risk of pathogens. It has been found that *Bifidobacterium* promotes health by eliminating

procarcinogens (Reddy and Rivenson, 1993; Rastall and Gibson *et al.,* 2002), production of vitamins such as vitamin B_2, vitamin B_6 and biotin (Teraguchi *et al.*, 1984). It also produces antimicrobial compounds against harmful intestinal microbes (Gibson and Roberfroid, 1995; Marteau *et al.,* 2001), and improves the immune system (Mitsuoka, 1992). Probiotic action of *Bifidobacteria* can be accounted by the fact that they can colonize within the intestine to interact with the host (Yildirin and Johnson, 1998).

Bifidobacterium has been classified into six main phylogenetic clusters, *Bifidobacterium boum*, *Bifidobacterium asteroids*, *Bifidobacterium adolescentis*, *Bifidobacterium pullorum*, *Bifidobacterium longum* and *Bifidobacterium pseudolongum,* based on comparative analysis of 16S rRNA-encoding DNA and concatenated multilocus sequences, representing a number of conserved house-keeping genes such as *clpC, dnaJ, xfp, dnaB, rpoC and purF* respectively.

Miller *et al.,* 2015 have reported that the pathological post-mortem reports reveal 23.5% of deaths among Elephants are due to the microbial infection of gastro-intestinal tract. Among the reported pathogens, *Salmonella* and *E.coli*, the opportunistic pathogens inhabit the gut of these animals. The pathogens can enter the body through direct contact or through contaminated food and water (Wilson, 2008).

Food and Agriculture Organisation (FAO) and World Health Organisation (WHO), proposes that probiotic are the live organisms which when taken in requisite amount benefits the health of the host. The capacity of the bacteria to adhere to the intestinal mucosa and tolerance to bile and acid stress could be the specific criteria for the presence of probiotic strain. The property of the micro-organisms to adhere can be important for the immune system enabling the eradication of pathogens (Castegliuolo, *et al.,* 2005).

Motherway in 2011, discovered pili-like proteins, for the extracellular export of components in *B.breve*. He also found that Type IVb tight adherence (Tad) pilus encoding gene is essential for the adherence to intestine of the host. Tad gene is found

to be conserved among the *Bifidobacterium* genera, indicating their vital role in the functioning of microbes.

The proposed study aims at studying the probiotic properties of the *Bifidobacterium* isolates and to determine their role in the improvement of health. The probiotic bacteria may have clinical potential against diseases like colon cancer, necrotizing enterocolitis and inflammatory bowel response (Robles and Guarner, 2013). Expression profiling of probiotic attributes of *Bifidobacteria* under different environmental conditions with respect to the gastro-intestinal tract of the concerned animal will be studied. A further follow up of the study is the administration of prospective probiotic strains to patients with acute or chronic gut disorders via the *in vivo* experiments.

REVIEW OF LITERATURE

- **Nebra and Blanch, (1999)** found new selective medium for the isolation of *Bifidobacterium* spp. Medium was found to be, antibiotic-free, contained main source of carbon as lactulose, inhibitors of some related bacterial species as methylene blue, propionic acid and lithium chloride, had low pH which is responsible for the inhibition of growth of *Enterobacteriaceae*.
- **Amor, *et al.*, (2002)** analysed the viability and stability of probiotic bacteria, *Bifidobacterium lactis* and *Bifidobacterium adolescentis*, under deconjugated bile salts stress, through a method based on fluorescent probes.
- **Wasilewska and Bielecka, (2003)** isolated *Bifidobacterium* strains from breast-fed infant.
- **Bonjoch, *et al.*, (2004)** studied the presence and measurement of human *Bifidobacterium* spp. municipal sewage and in slaughter house wastewater.
- **Vlkova, *et al.*, (2004)** studied the isolation and enumeration of *Bifidobacterium* using modified TPY agar. Thereafter isolates were identified at genus level using FISH method and by detection of fructose-6-phosphate phosphoketolase.
- **Moubarack, *et al.*, (2005)** studied the effect of antimicrobial agents on *Bifidobacterium* spp. and analysed its antibiotic susceptibility.
- **Savijoki, *et al.*, (2005)** investigated protein synthesis in cells of *Bifidobacterium longum* before and after heat shock and bile salts treatment revealed through two-dimensional gel electrophoresis and [^{35}S] methionine labelling.
- **Zinedine and Faid, (2007)** did isolation, identification and characterization of *Bifidobacteria*. Identification was based on phenotypic properties, the carbohydrates fermentation profiles and Fructose-6-phosphoketolase test. Also, studied that some strains were able to inhibit the growth of pathogenic cells. Therefore, it could be revealed that bacteriocins –like compounds are being produced. Furthermore, strains depicted probiotic properties.

- **Fukuda, *et al.*, (2011)** proposed that the acetate production by *Bifidobacterium* may facilitate the epithelial cells to improve upon defence mechanism and thus protecting the host against deadly infections.
- **Motherway, *et al.*, (2011)** investigated the genome sequence of *Bifidobacterium breve* strain to understand the colonization of the bacterium in the host intestine. An in-vivo experiment was conducted in murine intestine for the expression of tad gene to study the adherence properties. It was confirmed through transmission electron microscopy that Tad pilli was present at the poles of *Bifidobacterium breve* UCC2003 cells.
- **Yu, *et al.*, (2012)** discovered complete genome sequence of 2.2Mb of the probiotic bacteria *Bifidobacterium bifidium* strain BGN4, isolated from the faecal samples of breast-fed infant.
- **Bunesova, *et al.*, (2013)** identified *Bifidobacteria boum* and *Bifidobacterium adolescentis* from Asian elephant (*Elephas maximus*) faeces through MALDI-TOF MS, PCR methods and sequencing.
- **Singh, *et al.*, (2013)** isolated cellulose degrading bacteria and cellulose hydrolytic bacteria from rhinoceros dung. Bacteria, *Bacillus amyloliquefaciens* SS35, isolate was confirmed by 16S rDNA and partial gyrase A gene sequencing.
- **Bottacini, *et al.*, (2014)** studied the diversity and ecology of *Bifidobacterium* on the basis of 16S rRNA-encoding DNA. Also, studied the molecular mechanisms that explain how the *Bifidobacteria* persist and colonize in the gastrointestinal tract and interact with the human host.
- **Yang, *et al.*, (2014)** isolated cellulolytic bacteria, *Bacillus subtilis*, from Tibetan pig's intestine and identified morphologically, physiologically, biochemically and through 16S rRNA analysis and found the bacterium to produce cellulose. It was concluded that the endogenous animal cellulose shortage can be overcome by the microbe and rate of roughage usage can be improvised.

- **Presti, *et al.*, (2015)** investigated the probiotic properties of *Lactobacillus* and *Bifidobacterium* strains by evaluating in vitro experiments on aspects such as tolerance to vitamins, senstivity to antibiotics, pH, bile salts, antimicrobials and inflammation response.

OBJECTIVES

1. Isolation and confirmation of *Bifidobacteria* from herbivores/ fermented foods/sewage.
2. Evaluation of the probiotic properties of *Bifidobacteria* in order to search for novel strains with probiotic potential.
3. Expression profiling of gene responsible for probiotic attributes of *Bifidobacterium*.

METHODOLOGY

1. Creating aseptic environment in the lab for the growth of bacteria.
2. Enrichment of *Bifidobacteria* in Wilkins-chalgren broth.
3. Isolation, detection and identification of *Bifidobacteria* on Wilkins Soya –Peptone-Mupirocin media (WSP mup) (Rada and Petr, 2000).
4. Confirmation of *Bifidobacteria* isolates
    i. Biochemically by employing
        a) Gram staining with methylene blue and observing under microscope and strains with "Y" morphology will be selected for further identification (Rasic and Kurman, 1983)
        b) Sugar tests, Catalase test etc.
    ii. Molecular characterization by 16S rRNA gene sequencing (Roy and Sirosis, 2000)
5. Determination of antimicrobial properties of the isolates against pathogens
    i. By well-diffusion method (Piddock, 1990) : pathogenic cells will be grown on Trypic Soya Agar and wells will be hollowed on the TSA plate and *Bifidobacterium* culture will be incorporated in these wells and clear zone diameter will be observed after 24hours of incubation of 37°C.
    ii. Detection of specific molecules such as conjugated linoleic acid (Coakley, *et al.,* 2003), LuxS (Carey and Kostrzynska, 2013), Tad pilli (Kachlany, *et al.,* 2001: Schreiner, *et al.,* 2003), surface exopolysaccharide (sEPS) (Ventura, *et al.,* 2007; Ruas-Madiedo, *et al.,* 2009) etc.
6. Expression of gene responsible for probiotic attributes like formation
    i. RNA extraction from bacteria- RNA will be extracted following the trizol protocol.
    ii. cDNA (complementary DNA) synthesis through RT-PCR (reverse transcriptase- PCR).

iii. Relative quantification of gene expression by Real-Time PCR.

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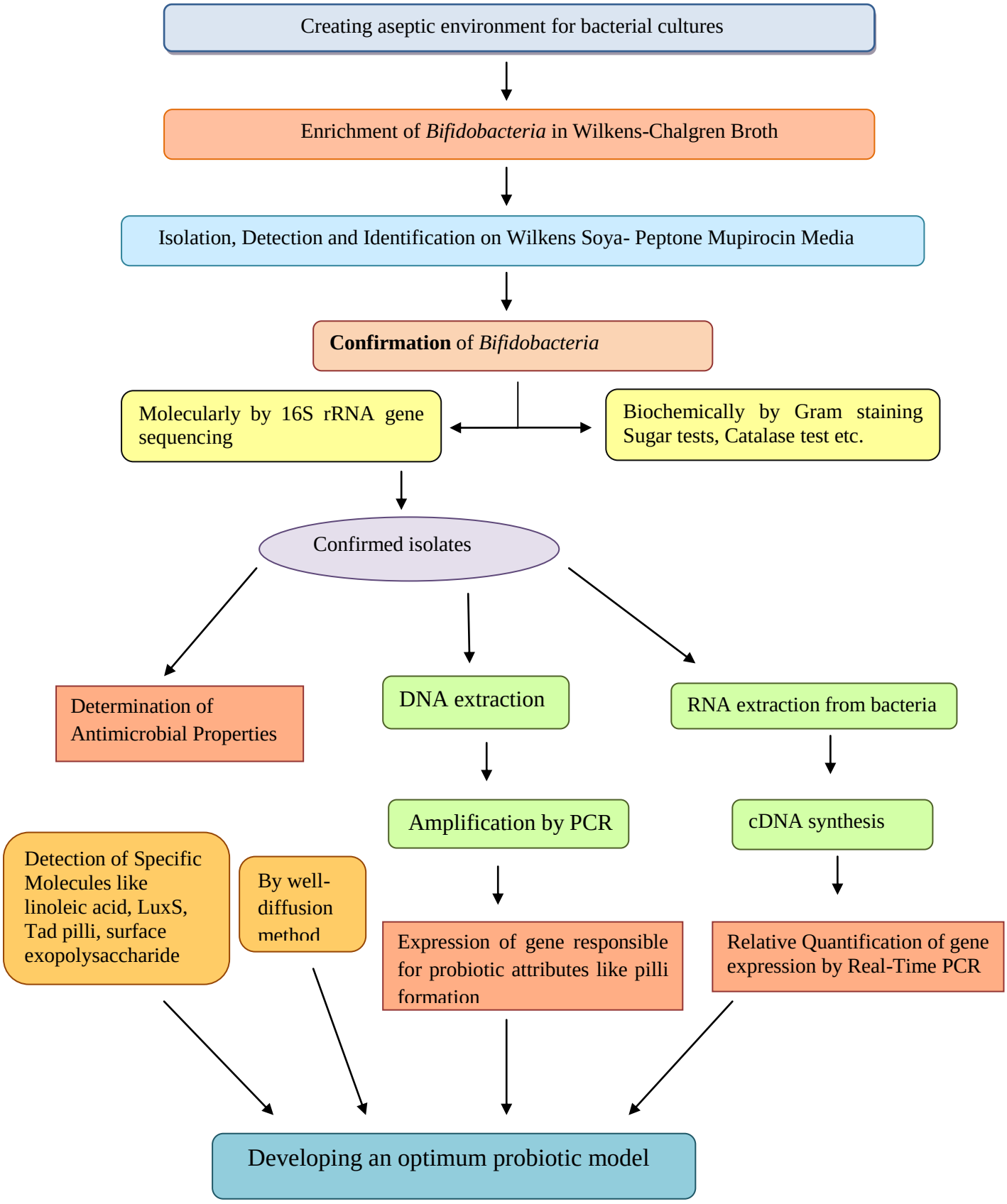
Plan of Work


Creating aseptic environment for bacterial cultures
Enrichment of *Bifidobacteria* in Wilkens-Chalgren Broth
Isolation, Detection and Identification on Wilkens Soya- Peptone Mupirocin Media
Confirmation of *Bifidobacteria*
Molecularly by 16S rRNA gene sequencing
Biochemically by Gram staining Sugar tests, Catalase test etc.
Confirmed isolates
Determination of Antimicrobial Properties
DNA extraction
RNA extraction from bacteria
Amplification by PCR
cDNA synthesis
Detection of Specific Molecules like linoleic acid, LuxS, Tad pilli, surface exopolysaccharide
By well-diffusion method
Expression of gene responsible for probiotic attributes like pilli formation
Relative Quantification of gene expression by Real-Time PCR
Developing an optimum probiotic model